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(54) Title: PLANT PATHOGEN RESISTANCE GENES AND USES THEREOF

(57) Abstract

The tomato *Cf-9* gene has been cloned and its sequence provided, along with the encoded amino acid sequence. DNA encoding the polypeptide, alleles, mutants and derivatives thereof, and DNA encoding amino acid sequences showing a significant degree of homology thereto may be introduced into plant cells and the encoded polypeptide expressed, conferring pathogen resistance on plants comprising such cells and descendants thereof. The CF-9 sequence comprises leucine rich repeats and the presence of such repeats enables identification of other plant pathogen resistance genes.

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PLANT PATHOGEN RESISTANCE GENES AND USES THEREOF

The present invention relates to pathogen resistance in plants and more particularly the identification and use of pathogen resistance genes.

Plants are constantly challenged by potentially pathogenic microorganisms. Crop plants are particularly vulnerable, because they are usually grown as genetically uniform monocultures; when disease strikes, losses can be severe. However, most plants are resistant to most plant pathogens. To defend themselves, plants have evolved an array of both preexisting and inducible defences. Pathogens must specialize to circumvent the defence mechanisms of the host, especially those biotrophic pathogens that derive their nutrition from an intimate association with living plant cells. If the pathogen can cause disease, the interaction is said to be compatible, but if the plant is resistant, the interaction is said to be incompatible. Race specific resistance is strongly correlated with the hypersensitive response (HR), an induced response by which (it is hypothesized) the plant deprives the pathogen of living host cells by localized cell death at sites of attempted pathogen ingress.

It has long been known that HR-associated disease resistance is often (though not exclusively) specified by dominant genes (*R* genes). Flor showed that when pathogens mutate to overcome such *R* genes, these mutations are recessive. Flor concluded that for *R* genes

to function, there must also be corresponding genes in the pathogen, denoted avirulence genes (*Avr* genes). To become virulent, pathogens must thus stop making a product that activates *R* gene-dependent defence mechanisms (Flor, 1971). A broadly accepted working hypothesis, often termed the elicitor/ receptor model, is that *R* genes encode products that enable plants to detect the presence of pathogens, provided said pathogens carry the corresponding *Avr* gene (Gabriel and Rolfe, 1990). This recognition is then transduced into the activation of a defence response.

Some interactions exhibit different genetic properties. *Helminthosporium carbonum* races that express a toxin (Hc toxin) infect maize lines that lack the *Hm1* resistance gene. Mutations to loss of Hc toxin expression are recessive, and correlated with loss of virulence, in contrast to gene-for-gene interactions in which mutations <u>to</u> virulence are recessive. A major accomplishment was reported in 1992, with the isolation by tagging of the *Hm1* gene (Johal and Briggs, 1992). Plausible arguments have been made for how gene-for-gene interactions could evolve from toxin-dependent virulence. For example, plant genes whose products were the target of the toxin might mutate to confer even greater sensitivity to the toxin, leading to HR, and the conversion of a sensitivity gene to a resistance gene. However, this does not seem to be the mode of action of *Hm1*, whose gene product inactivates Hc toxin.

Pathogen avirulence genes are still poorly understood. Several bacterial *Avr* genes encode hydrophilic proteins with no homology to other classes of protein, while others carry repeating units whose number can be modified to change the range of plants on which they exhibit avirulence (Keen, 1992; Long and Staskawicz, 1993). Additional bacterial genes (*hrp* genes) are required for bacterial *Avr* genes to induce HR, and also for pathogenicity (Keen, 1992; Long and Staskawicz, 1993). It is not clear why pathogens make products that enable the plant to detect them. It is widely believed that certain easily discarded *Avr* genes contribute to but are not required for pathogenicity, whereas other *Avr* genes are less dispensable (Keen, 1992; Long and Staskawicz, 1993). The characterization of one fungal avirulence gene has also been reported; the *Avr9* gene of *Cladosporium fulvum*, which confers avirulence on *C. fulvum* races that attempt to attack tomato varieties that carry the *Cf-9* gene, encodes a secreted cysteine-rich peptide with a final processed size of 28 amino acids but its role in compatible interactions is not clear (De Wit, 1992).

The technology for gene isolation based primarily on genetic criteria has improved dramatically in recent years, and many workers are currently attempting to clone a variety of *R* genes. Targets include (amongst others) rust resistance genes in maize, *Antirrhinum* and flax (by transposon tagging); downy mildew resistance genes in

lettuce and *Arabidopsis* (by map based cloning and T-DNA tagging); *Cladosporium fulvum* (*Cf*) resistance genes in tomato (by tagging, map based cloning and affinity labelling with avirulence gene products); virus resistance genes in tomato and tobacco (by map based cloning and tagging); nematode resistance genes in tomato (by map based cloning); and genes for resistance to bacterial pathogens in *Arabidopsis* and tomato (by map based cloning).

The map based cloning of the tomato *Pto* gene that confers "gene-for-gene" resistance to the bacterial speck pathogen *Pseudomonas syringae* pv tomato (Pst) has been reported (Martin *et al*, 1993). A YAC (yeast artificial chromosome) clone was identified that carried restriction fragment length polymorphism (RFLP) markers that were very tightly linked to the gene. This YAC was used to isolate homologous cDNA clones. Two of these cDNAs were fused to a strong promoter, and after transformation of a disease sensitive tomato variety, one of these gene fusions was shown to confer resistance to Pst strains that carry the corresponding avirulence gene, *AvrPto*. These two cDNAs show homology to each other. Indeed, the *Pto* cDNA probe reveals a small gene family of at least six members, 5 of which can be found on the YAC from which *Pto* was isolated, and which thus comprise exactly the kind of local multigene family inferred from genetic analysis of other R gene loci.

The *Pto* gene cDNA sequence is puzzling for proponents of the simple elicitor/receptor model. It reveals unambiguous homology to serine/threonine kinases, consistent with a role in signal transduction Intriguingly, there is strong homology to the kinases associated with self incompatibility in Brassicas, which carry out an analogous role, in that they are required to prevent the growth of genotypically defined incompatible pollen tubes. However, in contrast to the Brassica SRK kinase (Stein *et al* 1991), the *Pto* gene appears to code for little more than the kinase catalytic domain and a potential N-terminal myristoylation site that could promote association with membranes. It would be surprising if such a gene product could act alone to accomplish the specific recognition required to initiate the defence response only when the *AvrPto* gene is detected in invading microrganisms. The race-specific elicitor molecule made by Pst strains that carry *AvrPto* is still unknown and needs to be characterized before possible recognition of this molecule by the Pto gene product can be investigated.

We have now isolated the tomato *Cf-9* gene which confers resistance against the fungus *Cladosporium fulvum* and we have sequenced the DNA and deduced the amino acid sequence from this gene. The DNA sequence of the tomato *Cf-9* genomic gene is shown in SEQ ID NO.1 (and Figure 2) and the deduced amino acid sequence is shown in SEQ ID NO. 2 (and Figure 3). A cDNA sequence is shown in SEQ ID

NO. 4 (and Figure 4).

As described in more detail below, the tomato *Cf-9* gene was isolated by a method which involved use of a transformed line of tomato engineered for expression of the *Avr9* avirulence gene. This transformed line, which constitutively expressed functional, mature Avr9 protein, was crossed to plants which carried the *Cf-9* gene so that a proportion of the progeny exhibited a necrotic phenotype culminating in seedling death. The *Cf-9* gene was identified by the technique of transposon tagging with tagging of the *Cf-9* gene being confirmed by survival of the resulting seedlings.

According to one aspect, the present invention provides a DNA isolate encoding a pathogen resistance gene or a fragment thereof, the gene being characterized in that it encodes the amino acid sequence shown in SEQ ID NO 2 or an amino acid sequence showing a significant degree of homology thereto.

For example, the DNA isolate comprises DNA encoding an amino acid sequence showing 60% homology, preferably 80% homology, more preferably 90% homology to the amino acid sequence shown in SEQ ID NO 2. Most preferably the DNA encodes the amino acid sequence shown in SEQ ID NO 2 in which case the DNA isolate may comprise DNA having the sequence shown in SEQ ID NO 1 or SEQ ID NO 4, or part of either of these sufficient to encode the desired polypeptide (eg from the initiating methionine codon to the first in frame downstream stop codon). In one

embodiment the DNA comprises a sequence of nucleotides which are the nucleotides 1871 to 2969 of SEQ ID NO 1, or a mutant, derivative or allele thereof. A further aspect of the invention provides a DNA isolate encoding a pathogen resistance gene, or a fragment thereof, obtainable by screening a DNA library with a probe comprising nucleotides 1871 to 2969 of SEQ ID NO 1, or a fragment, derivative, mutant or allele thereof, and isolating DNA which encodes a polypeptide able to confer pathogen resistance to a plant, such as resistance to *Cladosporium fulvum (eg. expressing Avr9)*. The plant may be tomato. Suitable techniques are well known in the art.

DNA according to the present invention may encode the amino acid sequence shown in SEQ ID NO 2 or a mutant, derivative or allele of the sequence provided. Preferred mutants, derivatives and alleles are those which retain a functional characteristic of the protein encoded by the wild-type gene, especially the ability to confer pathogen resistance. Changes to a sequence, to produce a mutant or derivative, may be by one or more of insertion, deletion or substitution of one or more nucleotides in the nucleic acid, leading to the insertion, deletion or subsitution of one or more amino acids. Of course, changes to the nucleic acid which make no difference to the encoded amino acid sequence are included.

The DNA isolate, which may contain the DNA encoding the amino acid sequence of SEQ ID NO 2 or an amino acid

sequence showing a significant degree of homology thereto as genomic DNA or cDNA, may be in the form of a recombinant vector, for example a phage or cosmid vector. The DNA may be under the control of an appropriate promoter and regulatory elements for expression in a host cell, for example a plant cell. In the case of genomic DNA, this may contain its own promoter and regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter and regulatory elements for expression in the host cell.

Those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation seuqences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press.

When introducing a chosen gene construct into a cell, certain considerations must be taken into account, well known to those skilled in the art. The nucleic acid to be inserted should be assembled within a construct which contains effective regulatory elements which will drive transcription. There must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into

the endogenous chromosomal material may or may not occur according to different embodiments of the invention. Finally, as far as plants are concerned the target cell type must be such that cells can be regenerated into whole plants.

Plants transformed with the DNA segment containing pre-sequence may be produced by standard techniques which are already known for the genetic manipulation of plants. DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by Agrobacterium exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215 1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966), electroporation (EP 290395, WO 8706614) or other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611). Agrobacterium transformation is widely used by those skilled in the art to transform dicotyledonous species. Although Agrobacterium has been reported to be able to transform foreign DNA into some monocotyledonous species (WO 92/14828), microprojectile bombardment, electroporation and direct DNA uptake are preferred where Agrobacterium is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, eg. bombardment with Agrobacterium coated microparticles (EP-A-486234) or

mircoprojectile bombardment to induce wounding followed by co-cultivation with Agrobacterium (EP-A-486233).

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention.

The *Cf-9* gene and modified versions thereof encoding a protein showing a significant degree of homology to the protein product of the *Cf-9* gene, alleles, mutants and derivatives thereof, may be used to confer resistance in plants, in particular tomatoes, to a pathogen such as *C. fulvum*. For this purpose a vector as described above may be used for the production of a transgenic plant. Such a plant may possess pathogen resistance conferred by the *Cf-9* gene.

The invention thus further encompasses a host cell transformed with such a vector, especially a plant or a microbial cell. Thus, a host cell, such as a plant cell, comprising nucleic acid according to the present invention is provided. Within the cell, the nucleic acid may be incorporated within the chromosome.

A vector comprising nucleic acid according to the present invention need not include a promoter,

particularly if the vector is to be used to introduce the nucleic acid into cells for recombination into the genome.

Also according to the invention there is provided a plant cell having incorporated into its genome a sequence of nucleotides as provided by the present invention, under operative control of a promoter for control of expression of the encoded polypeptide. A further aspect of the present invention provides a method of making such a plant cell involving introduction of a vector comprising the sequence of nucleotides into a plant cell. Such introduction may be followed by recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome. The polypeptide encoded by the introduced nucleic acid may then be expressed.

Plants which comprise a plant cell according to the invention are also provided, along with any part or clone thereof, seed, selfed or hybrid progeny and descendants.

The invention further provides a method of comprising expression from nucleic acid encoding the amino acid sequence SEQ ID NO 2, or a mutant, allele or derivative thereof, or a significantly homologous amino acid sequence, within cells of a plant (thereby producing the encoded polypeptide), following an earlier step of introduction of the nucleic acid into a cell of the plant or an ancestor thereof. Such a method may confer pathogen resistance on the plant.

A gene stably incorporated into the genome of a plant is passed from generation to generation to descendants of the plant, cells of which decendants may express the encoded polypeptide and so may have enhanced pathogen resistance. Pathogen resistance may be determined by assessing compatibility of a pathogen (eg. *Cladosporium fulvum* or using recombinant expression of a pathogen avirulence gene, such as *Avr9*. Such a gene may be introduced into cells of a plant by any suitable transformation technique or by cross-breeding, as discussed herein.

Sequencing of the *Cf-9* gene has shown that it includes DNA sequence encoding leucine-rich repeat (LRR) regions and homology searching has revealed strong homologies to other genes containing LRRs. For the reasons discussed in more detail below, the presence of LRRs can be hypothesised to be characteristic of many pathogen resistance genes and the presence of LRRs can thus be used in a method of identifying further pathogen resistance genes.

According to a further aspect, the present invention provides a method of identifying a plant pathogen resistance gene which comprises:

(1) obtaining expressed or genomic DNA from cells of a plant possessing resistance to a pathogen;

(2) sequencing the DNA and identifying putative pathogen resistance genes by the presence of LRRs;

and

(3) confirming identification as a pathogen resistance gene.

DNA which may contain a pathogen resistance gene can be obtained in many ways. In the course of map-based cloning of disease resistance genes, genetic analysis may identify YAC clones that may possibly carry the resistance gene. Such YAC clones could then be used to screen cDNA clones from a cDNA library, and homologous cDNA clones that mapped from the region sequenced. These sequences can then be inspected for the presence of LRRs and putative pathogen resistance genes identified on the basis of such LRRs.

Alternatively, random DNA sequences from an appropriate plant source can be obtained, for example as cDNA or as genomic DNA in a cosmid vector or YAC, and this random DNA can be sequenced and putative pathogen resistance genes identified on the basis of LRRs. A large amount of DNA sequence information has already been generated from DNA derived from many different sources and this sequence information is available in databases. Such known DNA sequences can be searched for LRRs and sequence from an appropriate source showing LRRs can again be identified as a putative pathogen resistance gene.

LRRs are already known in many different genes (see for example Chang *et al* 1992) so that sequences of this type can readily be identified. Identification of LRRs

can be by simple visual inspection of the sequence to find areas of sequence that carry repeated motifs that are rich in leucine residues. Alternatively an appropriate computer searching technique can be used to determine homology to a known sequence containing LRRs or to a consensus sequence derived from known sequences containing LRRs. More particularly, use can be made of one or other of the various available algorithms for local sequence similarity searching such as BLASTX. Thus, for example, a BLASTX search can be used in databases at the US National Center for Biological Information and an LRR containing sequence can be identified by a BLASTX score of at least 60 or more against the sequence for *Cf-9* as set out in SEQ ID NO 2.

Once a putative pathogen resistance gene has been identified, this can be investigated further, if necessary following isolation of the full coding sequence, by linkage analysis to determine the chromosome on which the gene is located and whether it is linked to known locations for pathogen resistance genes. Such linkage analysis may also give indications as to the nature of the pathogen involved. Following linkage analysis, identification of a pathogen resistance gene can be confirmed by reintroduction of the DNA back into a plant with an appropriate genotype and investigation of the effect of that DNA on the transformed plant. If the effect is to confer resistance to a specific pathogen to an otherwise non-resistant plant, then this confirms the

gene as a pathogen resistance gene.

The techniques described above are of general applicability to the identification of pathogen resistance genes in plants. Examples of the type of genes that can be identified in this way include *Phytophthora* resistance in potatoes, mildew resistance and rust resistance in cereals such as barley and maize, rust resistance in *Antirrhinum* and flax, downy mildew resistance in lettuce and *Arabidopsis*, virus resistance in potato, tomato and tobacco, nematode resistance in tomato, resistance to bacterial pathogens in *Arabidopsis* and tomato and *Xanthomonas* resistance in peppers.

Once a pathogen resistance gene has been identified, it can be reintroduced into the plant in question by techniques well known to those skilled in the art to produce transgenic plants that have been engineered to carry the resistance gene in question. According to a further aspect, the present invention provides a DNA isolate encoding the protein product of a plant pathogen resistance gene which has been identified by use of the presence therein of LRRs and, in particular, by the technique defined above. According to a yet further aspect, the invention provides transgenic plants, in particular crop plants, which have been engineered to carry pathogen resistance genes which have been identified by the presence of LRRs. Examples of suitable plants include tobacco, cucurbits, carrot, vegetable brassica, lettuce, strawberry, oilseed

brassica, sugar beet, wheat, barley, maize, rice, soyabeans, peas, sorghum, sunflower, tomato, potato, pepper, chrysanthemum, carnation, poplar, eucalyptus and pine.

Further aspects and embodiments of the patent invention will be apparent to those skilled in the art. All documents mentioned herein are incorporated by reference.

As already indicated, the present invention is based on the cloning and sequencing of the tomato *Cf-9* gene and this experimental work is described in more detail below with reference to the following figures.

Figure 1 shows a schematic representation of the *Cf-9* gene.

Figure 2 shows the genomic DNA sequence of the *Cf-9* gene (SEQ ID NO 1). Features: Nucleic acid sequence - Translation start at nucleotide 898; translation stop at nucleotide 3487; polyadenylation signal (AATAAA) at nucleotide 3703-3708; polyadenylation site at nucleotide 3823; a 115 bp intron in the 3' non-coding sequence from nucleotide 3507/9 to nucleotide 3622/4. Predicted Protein Sequence - primary translation product 863 amino acids; signal peptide sequence amino acids 1-23; mature peptide amino acids 24-863.

Figure 3 shows Cf-9 protein amino acid sequence (SEQ ID NO 2).

Figure 4 shows the sequence of one of the CF9 cDNA

clones (SEQ ID NO 4). Translation initiates at the ATG at position +58.

Figure 5 shows a physical map of the tomato *Cf-9* locus generated from overlapping cosmids (34, 41, 110 and 138) isolated from the *Cf-2/Cf-9* cosmid library. The extent of each cosmid and location of the Cf-9 gene are shown schematically. Also indicated are the direction of the transcription (arrow) and the location of sites for restriction enzyme *Bgl*II (B).

Cloning of the tomato Cf-9 gene

As already indicated, the *C. fulvum* AVR9 gene and product are known (De Wit, 1992; van Kan et al 1991; Marmeisse *et al* 1993; Van Den Ackerveken *et al* 1993), Accordingly isolation of the *Cf-9* gene would be scientifically attractive, because it should enable characterization of binding between the AVR9 gene product ligand and the presumed *Cf-9* gene product receptor.

(i) Assignment of *Cf-* gene map locations.

We have mapped several *Cf* genes, including *Cf-9*, to their chromosomal locations (Dickinson et al 1993; Jones *et al* 1993; Balint-Kurti *et al* 1993). We showed that *Cf-4* and *Cf-9* map to approximately the same location on the short arm of chromosome 1, and *Cf-2* and *Cf-5* map to approximately the same location on chromosome 6. Others independently mapped *Cf-9* to chromosome 1 (van der Beek *et al* 1992).

(ii) Establishing transposon tagging in tomato

We have been establishing the capacity to carry out transposon tagging in tomato using the maize transposon *Activator* (*Ac*) and its *Dissociation* (*Ds*) derivatives (Scofield *et al* 1992; Thomas *et al* 1994; Carroll *et al* 1995). The strategy is founded on the fact that these transposons preferentially transpose to linked sites. Accordingly we have made available lines that carry *Dss* at positions that are useful to our colleagues. J Hille made available a line, FT33 (Rommens *et al* 1992), carrying a *Ds* linked to *Cf-9*. We have independently generated our own lines that carry a construct SLJ10512 (Scofield et al 1992) which contains (a) a beta-glucuronidase (*GUS*) gene (Jefferson *et al* 1987) to monitor T-DNA segregation and (b) stable *Ac* (*sAc*) that expresses transposase and can trans-activate a Ds, but which will not transpose (Scofield *et al* 1992).

(iii) Establishing a stock from which gametes carrying a mutagenized *Cf-9* gene could be obtained and identified

The line FT33 did not carry a *Cf-9* gene. We had to obtain recombinants that placed *Cf-9 in cis* with the T-DNA in FT33 in order to carry out linked targeted tagging. Two strategies were pursued simultaneously.

(a) FT33 was crossed to Cf9, a stock that carries the *Cf-9* gene. The resulting F1 was then back crossed to Cf0 (a stock that carries no *Cf-* genes). Progeny that carry the FT33 T-DNA are kanamycin resistant. Kanamycin

resistant progeny were tested for the presence of *Cf-9*; 5 *C. fulvum* resistant individuals were obtained among 180. We also generated progeny that were homozygous for *Cf-9* and carried the *sAc* T-DNA of SLJ10512. These were crossed to the recombinants in which *Cf-9* and FT33 were *in cis*. In the FT33 T-DNA, a transposable *Ds* element is cloned into a hygromycin resistance gene, preventing its function. The somatic transactivation of this *Ds* element, which only occurs in the presence of transposase gene expression, results in activation of the hygromycin resistance. Thus from crossing the recombinants between *Cf-9* and FT33, to the *sAc*-carrying *Cf-9* homozygotes, hygromycin resistant individuals could be obtained which carry *sAc* and FT33, and are likely to be homozygous for *Cf-9*. 140 individuals of this genotype were thus obtained.

(b) To accelerate obtaining individuals that carried *sAc*, FT33, and were *Cf-9* homozygotes, the FT33/*Cf-9* F1 was crossed to a line that was heterozygous for *Cf-9* and *sAc*. 25% of the resulting progeny carried both T-DNAs and were hygromycin resistant, and of those, slightly more than 50% were disease resistant because they carried at least one copy of the *Cf-9* gene. A restriction fragment length polymorphism (RFLP) marker was available, designated CP46, that enabled us to distinguish between homozygotes and heterozygotes for the *Cf-9* gene (Balint-Kurti *et al* 1994 (in press)). In this manner two individuals that were *Cf-9* homozygotes, and

that carried both the FT33 T-DNA and sAc, were obtained. These two individuals were multiplied by taking cuttings so that more crosses could be made onto this genotype.

(iv) Establishing a tomato stock that expresses functional mature AVR9 protein

A likely frequency for obtaining any desired mutation in a gene tagging experiment is less than 1 in 1000, and often less than 1 in 10,000 (Döring, 1989). To avoid screening many thousands of plants for mutations to disease sensitivity, we established a selection for such mutations based on expressing the fungal *Avr9* gene in plants. The sequence of the 28 amino acids of the mature Avr9 protein is known (van Kan *et al* 1991). It is a secreted protein and can be extracted from intercellular fluid of leaves infected with Avr9-carrying races of *C. fulvum*. For secretion from plant cells, we designed oligonucleotides to assemble a gene that carried a 30 amino acid plant signal peptide, from the Pr1a gene (Cornelissen *et al* 1987) preceding the first amino acid of the mature Avr9 protein (see SEQ ID No. 3). The preferred *Avr9* gene sequence depicted in SEQ ID No. 3 is a chimaeric gene engineered from the Pr-1a signal peptide sequence (Cornelissen *et al* 1987) and the *Avr9* gene sequence (van Kan *et al*, 1991). This reading frame was fused to the 35S promoter of cauliflower mosaic virus (Odell *et al* 1984), and the 3' terminator sequences of the octopine synthase gene (DeGreve *et al* 1983), and

introduced into binary plasmid vectors for plant transformation, using techniques well known to those skilled in the art, and readily available plasmids (Jones *et al* 1992). We obtained transformed Cf0 tomato lines that expressed this gene. These transformed lines were crossed to plants that carried the *Cf-9* gene. When the resulting progeny were germinated, 50% exhibited a necrotic phenotype, that culminated in seedling death. This outcome was only observed in seedlings that contained the *Avr9* gene. When the same transformants were crossed to Cf0 plants, the resulting prgeny were all fully viable. From selfing the primary transformants, individuals were identified that were homozygous for the *Avr9* transgene. When *Avr9* homozygotes were crossed to *Cf-9*, all progeny died. This system thus provides a powerful selection for individuals that carry mutations in the *Cf-9* gene (Hammond-Kosack et al 1994).

(v) Tagging the *Cf-9* gene

Individuals that were homozygous for the *Avr9* gene (section (iv)) were used as male parents to pollinate individuals that were homozogyous for *Cf-9*, and carried both s*Ac* and the *Ds* in the FT33 T-DNA (section (iiia) and (iiib)). Many thousands of progeny resulting from such a cross were germinated. Most died, but some survived.

DNA was obtained from survivors and subjected to Southern blot analysis using a *Ds* probe. It was observed that several independent mutations were correlated with

insertions of the *Ds* into a BglII fragment of a consistent size. The same result was observed with XbaI. This sugested that several independent mutations were a consequence of insertion of the *Ds* into the same DNA fragment.

Using primers to the *Ds* sequence, DNA adjacent to the *Ds* in transposed *Ds*-carrying mutant #18 was amplified using inverse PCR (Triglia *et al* 1988). This DNA was be used as a probe to other mutants, and proved that in independent mutations, the *Ds* had inserted into the same 6.7 kb BglII fragment.

The *Ds* in FT33 contains a bacterial replicon and a chloramphenicol resistance gene as a bacterial selectable marker (Rommens *et al* 1992). This means that plant DNA carrying this transposed *Ds* can be digested with a restriction enzyme that does not cut within the *Ds* (such as BglII), the digestion products can be recircularized, and then used to transform *E. coli*. Chloramphenicol resistant clones can be obtained that carry the *Ds* and adjacent plant DNA. This procedure was used to obtain a clone that carried 1.7 kb of plant DNA on the 3′ side of the *Ds*, and 4.9 kb of plant DNA on the 5′ side of the *Ds*.

Our current understanding of the *Cf-9* gene is depicted schematically in Figure 1. The 1.8 kb of plant DNA on the 3′ side of Ds extend between insertion #18 and the Bglll site on this figure. Further clones were obtained by digesting plant DNA of mutant #18 with Xbal instead of Bglll prior to recircularization and

transformation. This permitted the isolation of clones carrying DNA that extended considerably (at least 5kb) to the right of this Bglll site, and thus permitted sequencing of DNA to the right of the Bglll site shown in Figure 1.

Using a combination of various subclones, synthesis of new sequencing primers for further sequence determination based on newly established sequence (primers F1, 2, 3, 4, 5, 6, 7, 12, 13, 10, 26, 27 and 25 that were used in such experiments are indicated in the Figure), and other techniques well known to those skilled in the art, 3847 bp of sequence were determined. Various other restriction sites (Xhol, Sstl, EcoRI and Hindlll) are also indicated in Figure 1.

The F-series of primers were used to characterise a large number of independent mutations by PCR analysis in combination with primers based on the sequence of Ds. Therefore, these primers were used in polymerase chain reactions with primers based on the maize Ac/Ds transposon sequence, to characterise the locations of other mutations of *Cf-9* that were caused by transposon insertion.

Eighteen independent insertions were characterized and are located as shown. Mutants E, #55, #74 and #100 gave incomplete survival and showed a necrotic phenotype, and based on the available sequence information, they are 5′ to the actual reading frame and might permit enough Cf9 protein expression to activate an incomplete defence

response.

(vi) DNA sequence analysis of the *Cf-9* gene

DNA sequence analysis of the *Cf-9* gene has now been completed and upon conceptual translation has revealed an interesting motif (the leucine rich repeat, or LRR) that can be hypothesized to be diagnostic of other resistance genes. The genomic DNA sequence of *Cf-9* is shown in Figure 2 and SEQ ID NO. 1. Approximately 3.9 kbp of genomic DNA sequence has been determined. A translation start codon (ATG) sequence is located at position 898 and a translation termination codon TAG sequence is located at position 3487 bp (Figure 2), with an intervening uninterrupted 863 amino acid open reading frame.

Using the sequence obtained, oligonucleotide primers were designed that could be used in PCR reactions in combination with primers based on the sequence of the Ds element, to characterize both the location and the orientation of other transposon insertions in the gene. Based on the results of such experiments, the map positions of 17 other Ds insertions can now be reliably assigned (as shown in Figure 1).

The fact that 18 independent mutants that survive in the presence of Avr9, are associated with insertions into the same region of DNA, provides compelling evidence that the *Cf-9* gene has been tagged, and that DNA sequence obtained from this region is derived from the *Cf-9* gene.

Further proof is provided by the fact that when mutant # 18, (a stable mutant that lacks sAc) is back-crossed to a line homozygous for sAc, one quarter of the resulting progeny carry *sAc*, *Ds* # 18, and the Avr9 gene. These progeny exhibit variegation for a necrosis, consistent with the idea that on *sAc* dependent somatic excision of *Ds*, *Cf-9* gene function is somatically restored, leading to sectors that die.

Further proof is provided by the fact that individuals that survived the Avr9 selection lost disease resistance to races of *C. fulvum* that carry the Avr9 gene (Jones et al. 1994).

(vii) Identification of a leucine-rich repeat region in Cf-9.

The genomic DNA sequence of the *Cf-9* gene is shown in Figure 2 (SEQ ID NO. 1). The deduced amino acid sequence of the Cf-9 protein is shown in Figure 3 (SEQ ID NO. 2). Currently the 18 independent *Ds* insertions are all in or 5' to the 863 amino acid open reading frame shown in Figure 3. A cDNA library was constructed from messenger RNA isolated from tomato cotyledons injected with intercellular fluid containing AVR9 peptide in a bacteriophage lambda cloning vector. 600,000 cDNA clones were screened and 18 clones were identified that hybridized to DNA probes from sequences adjacent to the *Ds* insertions in the *Cf-9* gene. While some of these cDNA clones were from other members of the *Cf-9* multigene

family (Jones et al 1994), six clones were identified that are derived from the genomic sequence shown in Figure 2 because they show identical DNA sequence apart from the splicing out of a small intron in the 3' untranslated region between nucleotides 3509 and 3623 of the Figure 2 sequence. The sequence of one such cDNA clone is shown below in Figure 4 (SEQ ID NO. 4).

Homology searching of the resulting sequence against sequences in the databases at the US National Centre of Biological Information (NCBI) reveals strong homologies to other genes that contain leucine rich repeat regions (LRRs). These include the Arabidopsis genes TMK1 (Chang at al 1992), TMKL1 (Valon et al 1993), RLK5 (Walker, 1993), as well as expressed sequences with incomplete sequence and unknown function (e.g. Arabidopsis thaliana transcribed sequence [ATTS] 1447). The presence of LRRs has been observed in other genes, many of which probably function as receptors (see Chang et al (1992) for further references).

The TMK1 and RLK5 genes have structures which suggest they encode transmembrane serine/threonine kinases and carry extensive LRR regions. As yet no known function has been assigned to them. Disease resistance genes are known to encode gene products which recognize pathogen products and subsequently initiate a signal transduction chain leading to a defence response. It is known that another characterized disease resistance gene (*Pto*) is a protein kinase (Martin et al 1993). However,

in *Cf-9* there is no apparent protein kinase domain based on genomic DNA and cDNA sequence analysis.

The predicted Cf-9 amino acid sequence can be divided into 7 domains (see also figure 3 in Jones et al 1994):

Domain A is a 23 amino acid probable signal peptide.

Domain B is a 68 amino acid region with some homology to polygalacturonase inhibitor proteins.

Domain C is a 668 amino acid comprising 28 imperfect copies of a 24 amino acid leucine rich repeat (LRR).

Domain D is a 28 amino acid domain with some homology to polygalacturonase inhibitor proteins.

Domain E is a 18 amino acid domain rich in negatively charged residues.

Domain F is a 37 amino acid hydrophobic domain encoding a putative transmembrane domain.

Domain G is a 21 amino acid domain rich in positively charged residues.

Domains E, F and G together comprise a likely membrane anchor.

(viii) Isolation of binary cosmid vector clones that carry a genomic *Cf-9* gene

In order to demonstrate that the gene characterized by transposon tagging is indeed*Cf-9*, we have demonstrated that homologous DNA sequences from the Moneymaker Cf9

near isogenic line (the Cf9 stock) could confer both resistance to *C. fulvum* and sensitivity to Avr9 peptide in transgenic Cf0 tomato plants into which these sequences have been transformed.

A genomic DNA library was constructed from a stock that carried both the *Cf-9* gene on chromosome 1, and the *Cf-2* gene on chromosome 6, so that the library could be used for isolating both genes. The library was constructed in a binary cosmid cloning vector pCLD04541, obtained from Dr C. Dean, John Innes Centre, Colney Lane, Norwich (see also Bent et al., 1994). This vector is essentially similar to pOCA18 (Olszewski et al., 1988). It contains a bacteriophage lambda cos site to render the vector packageable by lambda packaging extracts and is thus a cosmid (Hohn and Collins, 1980). It is also a binary vector (van den Elzen et al., 1985), so any cosmid clones that are isolated can be introduced directly into plants to test for the function of the cloned gene.

High molecular weight DNA was isolated from leaves of 6 week old greenhouse-grown plants by techniques well known to those skilled in that art (Thomas *et al* 1994) and partially digested with MboI restriction enzyme. The partial digestion products were size fractionated using a sucrose gradient and DNA in the size range 20-25 kilobases (kb) was ligated to BamHI digested pCLD04541 DNA, using techniques well known to those skilled in the art. After *in vitro* packaging using Stratagene packaging extracts, the cosmids were introduced into a tetracycline

sensitive version (obtained from Stratagene) of the Stratagene *Escherichia coli* strain SURE™ . Recombinants were selected using the tetracycline resistance gene on pCLD04541.

The library was randomly distributed into 144 pools containing about 1500 clones per pool, cells were grown from each pool and from 10 ml of cells, 9 ml were used for bulk plasmid DNA extractions, and 1 ml was used after addition of 0.2 ml of glycerol, to prepare a frozen stock. Plasmid DNA was isolated by alkaline lysis (Birnboim and Doly, 1979), and was analyzed by PCR for pools that might carry *Cf-9* homologous DNA, using the PCR primers F7 and F10 with the sequences 5'GGAAGAGATGTTTACAGATTCAAGG3' (SEQ ID NO 5) and 5'ATCAGCAGGTCGATTCTTGTGG3' (SEQ ID NO 6) respectively, that prime towards each other from positions 707-728 and 1494-1518 of the genomic DNA sequence. Pools 34, 41, 110 and 138 proved to be positive by this assay.

For each pool, approximately 10,000 colonies were plated out and inspected for *Cf-9* homology by colony hybridization with a radioactive *Cf-9* probe, and from each pool, single clones were isolated that carried such homology and gave a PCR product upon carrying out a PCR reaction with the F7, F10, combination of primers. These techniques are all well known to those skilled in the art.

These clones have been further characterized by Southern blot hybridization using a *Cf-9* probe, and by

restriction enzyme mapping. Our current assessment of the extent of contiguous DNA around *Cf-9*, as defined by these overlapping cosmids is shown in Figure 5. These cosmids were subsequently used in plant transformation experiments, selecting for plant cells transformed to kanamycin resistance, using techniques well known to those skilled in the art. Transgenic tomato, tobacco and potato plants were produced (Fillatti et al., 1987; Hammond-Kosack et al., 1994; Horsch et al., 1985, Spychalla and Bevan, 1993) with at least one of each of cosmids 34, 41, 110 and 138.

(ix) Assessment of *Cf-9* function in transgenic tomato, tobacco and potato

The function of a putative cloned *Cf-9* gene can be assessed in transformed tomato by testing transformants not only for resistance to Avr9-carrying *C. fulvum*, but also for a necrotic response to intercellular fluid (IF) containing active Avr9 peptide. The function of a cloned *Cf-9* gene in species that are not a host for *C. fulvum* , such as tobacco and potato, can only be assessed by evaluating the response to IF.

To assess the biological activity conferred upon tomato, potato and tobacco primary transformants carrying different *Cf-9* cosmids, the interveinal panels of mature leaves were injected with IFs either containing or lacking Avr9 peptide. These IFs were prepared according to the procedure of de Wit and Spikman (1982). The IFs

containing Avr9 peptide were obtained from either a compatible *C. fulvum* interaction involving race 0 and Cf0 plants or transgenic tobacco plants homozygous for the 35S:Avr9 construct (SLJ 6201) (Hammond-Kosack et al. 1994). The IFs lacking Avr9 were obtained from either a compatible *C. fulvum* interaction involving race 2,4,5,9 and Cf0 plants or from untransformed tobacco plants.

A summary of the results from experiments with the various cosmids introduced into tomato, tobacco and potato is shown in Table 1. All the tomato plants that carried a functional *Cf-9* gene by the criterion of Avr9-induced necrosis, were also resistant to infection by *C. fulvum* races that express Avr9, unlike the *C. fulvum*- sensitive Cf0 Moneymaker variety into which the cosmid clone had not been transformed.

A *Cf-9* - *Avr9* - dependent grey necrotic response occurred within the IF injected leaf panels of most tomato (17 out of 23), potato (5 out of 5) and tobacco (10 out of 13) transformants by 24 hours post injection. These data indicate that the genomic *Cf-9* gene, under the control of its own promoter, is functional and exhibits the expected specificity of action when introduced into various plant species, including tomato, potato and tobacco.

Further confirmation of the biological activity of *Cf-9* in tobacco was obtained by crossing 5 different primary transformants carrying a single copy of cosmid 34 (transformed lines B, H, I, L and M), to transgenic

tobacco plants homozygous for the 35S:Avr9 T-DNA. Seedling lethality occurred in 50% of the F_1 progeny by 11 days after seed planting. A similar seedling lethal phenotype was obtained when tomato plants carrying *Cf-9* were crossed to 35S:Avr9 expressing tomato plants (Hammond-Kosack et al. 1994). These data demonstrate the feasibility of strategies that exploit the recognition between Avr9 and Cf-9 for engineering disease resistance in transgenic plants other than tomato.

TABLE 1

Plant Species	Trans'd Line	Cos'd #34	Cos'd #41	Cosmid #110	Cosmid #138
Tomato	A	+	+	+	+
	B	+	+	+	+
	C	-	+	+	+
	D	-	-	+	+
	E	-	+	-	+
	F	-	+		+
Potato	A			+	+
	B	+		+	+
Tobacco	A	-			
	B[1]	+[2]			
	C	-			
	D	+			
	E	+			
	F	+			
	G[1]	-			
	H[1]	+			
	I	+			
	J	+			
	K	+			
	L[1]	+			
	M[1]	+			

The response of transgenic tomato, potato and tobacco plants (primary transformants) carrying different *Cf-9* cosmid constructs to intercellular fluid containing Avr-9

peptide obtained from transgenic tobacco plants homozygous for the 35S::Avr9 constructs (SLJ 6201). A plus (+) indicates that grey necrotic symptoms formed within the injected leaf panel by 24hrs. A minus (-) indicates that there was no response. Copy numbers of cosmid inserts were determined by Southern blot analysis.

[1] Single copy of cosmid 34, used for crossing with transgenic tobacco plants homozygous for the 35S::Avr9 T-DNA.

[2] Plants also respond positively to IF containing Avr9 peptide obtained from a compatible *C.fulvum* interaction (race 0 - Cf0) but give no response to two different intercellular fluids lacking Avr9 (race 2,4,5,9 - Cf0) and untransformed tobacco.

KEY TO FIGURE 1

Figure 1 shows tagged alleles of the *Cf-9* gene. X is a probable promoter.

SEQ ID NO 3.

The amino acid sequence and DNA sequence of the preferred form of the chimaeric *Avr9* gene used as described herein.

```
ATG GGA TTT GTT CTC TTT TGA CAA TTG CCT TCA TTT CTT CTT GTC
M   G   F   V   L   F   S   Q   L   P   S   F   L   L   V

TCT ACA CTT CTC TTA TTC CTA GTA ATA TCC CAC TCT TGC CGT GCC
S   T   L   L   L   F   L   V   I   S   H   S   C   R   A

TAC TGT AAC AGT TCT TGT ACA AGA GCT TTT GAC TGT CTT GGA CAA
Y   C   N   S   S   C   T   R   A   F   D   C   L   G   Q

TGT GGA AGA TGC GAC TTT CAT AAG CTT CAA TGT GTA CAT TGA
C   G   R   C   D   F   H   K   L   Q   C   V   H
```

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Claims:

1. A DNA isolate encoding a pathogen resistance gene or fragment thereof, the gene being characterised in that it encodes the amino acid sequence shown in SEQ ID NO 2 or an amino acid sequence showing a significant degree of homology thereto.

2. A DNA isolate as claimed in claim 1 which encodes an amino acid sequence as shown in SEQ ID NO 2 or an allele, mutant or derivative thereof.

3. A DNA isolate as claimed in claim 2 which comprises DNA having the sequence shown in SEQ ID NO 1 or SEQ ID NO 4.

4. A recombinant vector in which DNA as claimed in any one of claims 1 to 3 is under control of an appropriate promoter and regulatory elements for expression in a host cell.

5. Use of a DNA isolate according to any one of claims 1 to 3 or a recombinant vector according to claim 4 for the production of a transgenic plant.

6. A host cell comprising a DNA isolate according to any one claims 1 to 3 or a recombinant vector according to claim 4.

7. A host cell according to claim 6 which is a microbial cell.

8. A host cell according to claim 6 which is a plant cell.

9. A plant or any part thereof comprising a plant cell according to claim 8.

10. Seed, selfed or hybrid progeny or a descendant of a plant according to claim 9, or any part thereof.

11. A method of conferring pathogen resistance on a plant, comprising expression from nucleic acid encoding the amino acid sequence shown in SEQ ID NO 2, or a mutant, allele or derivative thereof or a significantly homologous amino acid sequence, within cells of the plant, following an earlier step of introduction of the nucleic acid into a cell of the plant or an ancestor thereof.

12. A method according to claim 11 wherein the nucleic acid comprises the sequence shown in SEQ ID NO 1 or SEQ ID NO 4.

13. A method of identifying a plant pathogen resistance gene which comprises:

(1) obtaining expressed or genomic DNA from cells of a plant possessing resistance to a pathogen;

(2) sequencing the DNA and identifying putative pathogen resistance genes by the presence of leucine rich repeats (LRRs), and

(3) confirming identification as a pathogen resistance gene.

14. A method as claimed in claim 13 wherein LRRs are identified as having a BLASTX score of 60 or more when compared to the sequence of SEQ ID NO. 2.

15. A method according to claim 13 or claim 14 wherein identification as a pathogen resistance gene is confirmed by linkage analysis and/or the effect of the gene on the phenotype of an appropriate plant transformed therewith.

FIGURE 1

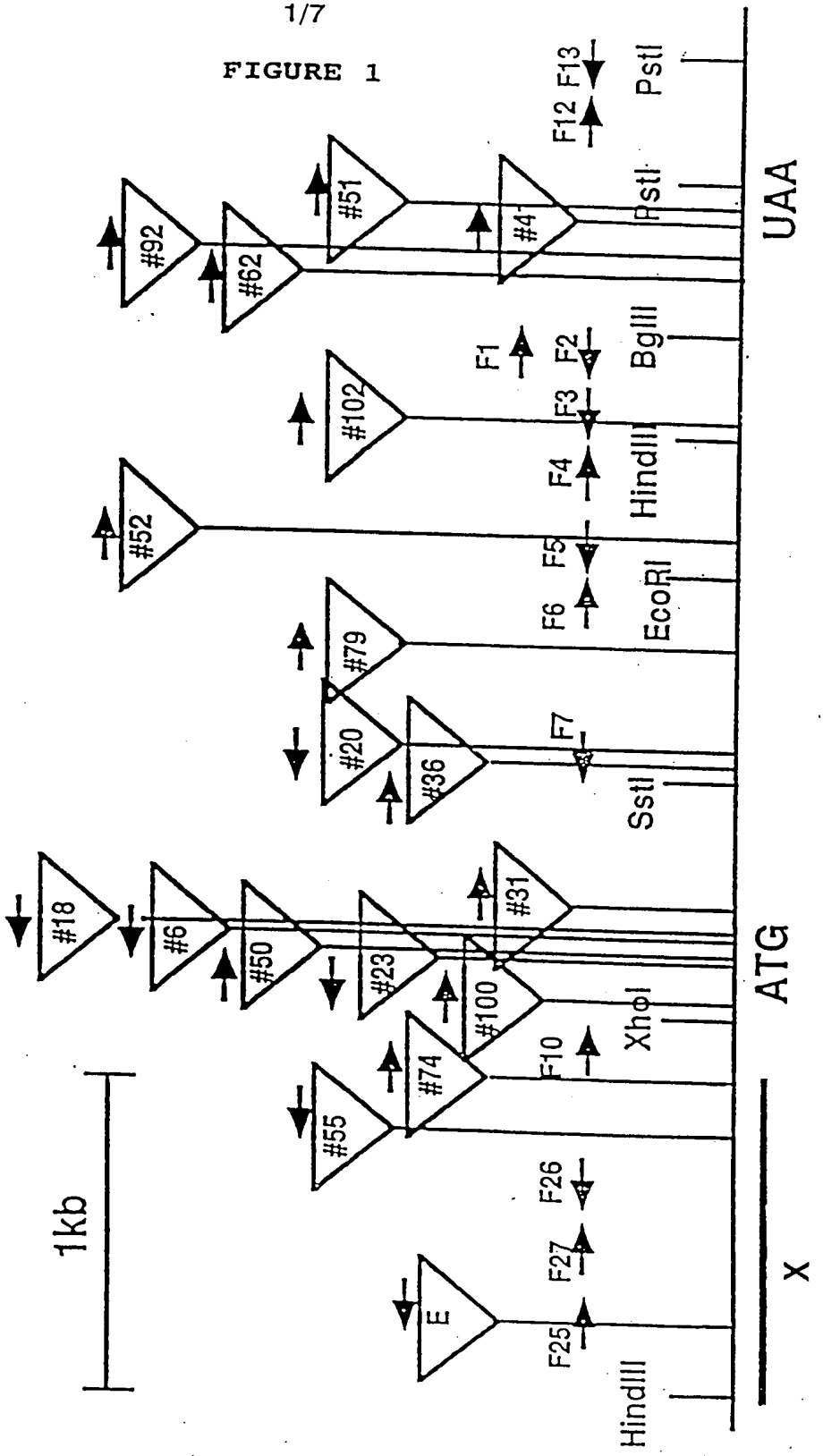
F13
PstI
F12
PstI
UAA
#51
#4
#92
#62
F1
F2
BglII
#102
F3
HindIII
F4
#52
F5
EcoRI
F6
#79
#20
#36
F7
SstI
#18
#6
#50
#23
#31
#100
XhoI
F10
ATG
#74
#55
F26
F27
E
F25
HindIII
1kb
X

FIGURE 2

```
1     CATAGTCTTT GCATATTTGG ATTAAACAGG GGCATTATTG AACCAAACTA
51    TTAGATGTAT GAAAATTTTG GACCAAGCTA TTGACAACAC GAACATTTTT
101   AGACCAAACT ATTAACTCAG AATATTTTCC GTTGAATGAA TAAGGTAACT
151   AGTAGTAAAT TTTTAGACCA AACTATGAAG AACATGCCAT GTCTGGACTC
201   CTGCACTATC TTCCATCAAC AGGTCAATTC TCTCAACTCT ATTGGTGGAA
251   GGTAGACGGT ACAAATTGAA TTATATTAAA AGACAAGCTC ACCTGAGCAT
301   CACTGTTATA CAACAACAAC AAACTACGCT TCAGCCCCAA ACAATAGTGA
351   CCCGAATCAT ATATTGTCAC GAGTTTTTTT TAGAGTATGT TGCATATATT
401   ATACTCAACT TAGGGTTTGT CATTCTGATG CTTCGTACAA ATTTATTGAA
451   TTTTCAACTT TAAAGGTTTA TGAACCAAAT ATTACGCTTA CTATGATAGC
501   GGTCTTTTTT GATTAATCAA ACTTATTGAA TTTTCAACTT TAAAGGTTTT
551   TCCCCGTTCT ATACACAAAC TAAGAAAAAT TTAAATTATA TAGTCTTTGG
601   ATGGTGACCT ATTTGGATGG TAACATTATT GGACCAAACT ATTGATAACG
651   CGGACATTGT TAGACCAAAC TGAGAAGGAC ATGTCTGGAC TCCTGCTCCG
701   TCTTCCATCA GCAGGTCGAT TCTTGTGGAA AATTAGCTCG AGGTGGCGCA
751   CTATGTGAGG TAACTAGTAC TAAATTTTTC TTTGCTTAAT TTGTGCTATA
801   TATACCTCAT CTAAATTATT GAATAGTCAC ACAAAGCAAA CATTTCTTGA
851   TTTCTTCTCT ATCAACATAA CAAGTTTTGA TCATTTTTAG TGCAGAAATG
901   GATTGTGTAA AACTTGTATT CCTTATGCTA TATACCTTTC TCTGTCAACT
951   TGCTTTATCC TCATCCTTGC CTCATTTGTG CCCCGAAGAT CAAGCTCTTT
1001  CTCTTCTACA ATTCAAGAAC ATGTTTACCA TTAATCCTAA TGCTTCTGAT
1051  TATTGTTACG ACATAAGAAC ATACGTAGAC ATTCAGTCAT ATCCAAGAAC
1101  TCTTTCTTGG AACAAAAGCA CAAGTTGCTG CTCATGGGAT GGCGTTCATT
1151  GTGACGAGAC GACAGGACAA GTGATTGCGC TTGACCTCCG TTGCAGCCAA
1201  CTTCAAGGCA AGTTTCATTC CAATAGTAGC CTCTTTCAAC TCTCCAATCT
1251  CAAAAGGCTT GATTTGTCTT TTAATAATTT CACTGGATCA CTCATTTCAC
1301  CAAAATTTGG TGAGTTTTCA AATTTGACGC ATCTCGATTT GTCGCATTCT
1351  AGTTTTACAG GTCTAATTCC TTCTGAAATC TGTCACCTTT CTAAACTACA
1401  CGTTCTTCGT ATATGTGATC AATATGGGCT TAGTCTTGTA CCTTACAATT
```

FIGURE 2 CONTINUED

1451 TTGAACTGCT CCTTAAGAAC TTGACCCAAT TAAGAGAGCT CAACCTTGAA
1501 TCTGTAAACA TCTCTTCCAC TATTCCTTCA AATTTCTCTT CTCATTTAAC
1551 AACTCTACAA CTTTCAGGCA CAGAGTTACA TGGGATATTG CCCGAAAGAG
1601 TTTTTCACCT TTCCAACTTA CAATCCCTTC ATTTATCAGT CAATCCCCAG
1651 CTCACGGTTA GGTTTCCCAC AACCAAATGG AATAGCAGTG CATCACTCAT
1701 GACGTTATAC GTCGATAGTG TGAATATTGC TGATAGGATA CCTAAATCAT
1751 TTAGCCATCT AACTTCACTT CATGAGTTGT ACATGGGTCG TTGTAATCTG
1801 TCAGGGCCTA TTCCTAAACC TCTATGGAAT CTCACCAACA TAGTGTTTTT
1851 GCACCTTGGT GATAACCATC TTGAAGGACC AATTTCCCAT TTCACGATAT
1901 TTGAAAAGCT CAAGAGGTTA TCACTTGTAA ATAACAACTT TGATGGCGGA
1951 CTTGAGTTCT TATCCTTTAA CACCCAACTT GAACGGCTAG ATTTATCATC
2001 CAATTCCCTA ACTGGTCCAA TTCCATCCAA CATAAGCGGA CTTCAAAACC
2051 TAGAATGTCT CTACTTGTCA TCAAACCACT TGAATGGGAG TATACCTTCC
2101 TGGATATTCT CCCTTCCTTC ACTGGTTGAG TTAGACTTGA GCAATAACAC
2151 TTTCAGTGGA AAAATTCAAG AGTTCAAGTC CAAAACATTA AGTGCCGTTA
2201 CTCTAAAACA AAATAAGCTG AAAGGTCGTA TTCCGAATTC ACTCCTAAAC
2251 CAGAAGAACC TACAATTACT TCTCCTTTCA CACAATAATA TCAGTGGACA
2301 TATTTCTTCA GCTATCTGCA ATCTGAAAAC ATTGATATTG TTAGACTTGG
2351 GAAGTAATAA TTTGGAGGGA ACAATCCCAC AATGCGTGGT TGAGAGGAAC
2401 GAATACCTTT CGCATTTGGA TTTGAGCAAA AACAGACTTA GTGGGACAAT
2451 CAATACAACT TTTAGTGTTG GAAACATTTT AAGGGTCATT AGCTTGCACG
2501 GGAATAAGCT AACGGGGAAA GTCCCACGAT CTATGATCAA TTGCAAGTAT
2551 TTGACACTAC TTGATCTAGG TAACAATATG TTGAATGACA CATTTCCAAA
2601 CTGGTTGGGA TACCTATTTC AATTGAAGAT TTTAAGCTTG AGATCAAATA
2651 AGTTGCATGG TCCCATCAAA TCTTCAGGGA ATACAAACTT GTTTATGGGT
2701 CTTCAAATTC TTGATCTATC ATCTAATGGA TTTAGTGGGA ATTTACCCGA
2751 AAGAATTTTG GGGAATTTGC AAACCATGAA GGAAATTGAT GAGAGTACAG
2801 GATTCCCAGA GTATATTTCT GATCCATATG ATATTTATTA CAATTATTTG
2851 ACGACAATTT CTACAAAGGG ACAAGATTAT GATTCTGTTC GAATTTTGGA
2901 TTCTAACATG ATTATCAATC TCTCAAAGAA CAGATTTGAA GGTCATATTC

FIGURE 2 CONTINUED

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2951 CAAGCATTAT TGGAGATCTT GTTGGACTTC GTACGTTGAA CTTGTCTCAC
3001 AATGTCTTGG AAGGTCATAT ACCGGCATCA TTTCAAAATT TATCAGTACT
3051 CGAATCTTTG GATCTCTCAT CTAATAAAAT CAGCGGAGAA ATTCCGCAGC
3101 AGCTTGCATC CCTCACATTC CTTGAAGTCT TAAATCTCTC TCACAATCAT
3151 CTTGTTGGAT GCATCCCCAA AGGAAAACAA TTTGATTCGT TCGGGAACAC
3201 TTCGTACCAA GGGAATGATG GGTTACGCGG ATTTCCACTC TCAAAACTTT
3251 GTGGTGGTGA AGATCAAGTG ACAACTCCAG CTGAGCTAGA TCAAGAAGAG
3301 GAGGAAGAAG ATTCACCAAT GATCAGTTGG CAGGGGGTTC TCGTGGGTTA
3351 CGGTTGTGGA CTTGTTATTG GACTGTCCGT AATATACATA ATGTGGTCAA
3401 CTCAATATCC AGCATGGTTT TCGAGGATGG ATTTAAAGTT GGAACACATA
3451 ATTACTACGA AAATGAAAAA GCACAAGAAA AGATATTAGT GAGTAGCTAT
3501 ACCTCCAGGT ATTCCACTTG ATCATTATCT TTCAGAAGAT TATTTTTTGT
3551 ATATCGATGA AATTATCGAC CTCCTTCATC CTCAAAGCTC TTAACTTTCA
3601 CTCTTCATTT TTGAAAATTT CAGGATTCAA AGATTTCCGA GTTCCCAGTT
3651 GCTTGGGATG CAGATAAAAG CCTTTTTATC TTTCATAGTT TCTTATCCTA
3701 TGAATAAAGA TTTTATTTTC ATTTGTCTAT GGCACGTAGA TATGTTCCGT
3751 CACTAAAAAC ATTGTATTTC TCTCAACTCT TTCGTCACAT GATATCAAAG
3801 AACACTTGAC TTCAATTAAG TTACTGTAGT CTGCTATTTT AATTTTTTCC
3851 ATTGAAACAC AACTGACGTA TCTTGAGAAA GAGACTATGA TCCCCCGGGC
3901 TGCAG
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FIGURE 3

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1    MDCVKLVFLM LYTFLCQLAL SSSLPHLCPE DQALSLLQFK NMFTINPNAS
51   DYCYDIRTYV DIQSYPRTLS WNKSTSCCSW DGVHCDETTG QVIALDLRCS
101  QLQGKFHSNS SLFQLSNLKR LDLSFNNFTG SLISPKFGEF SNLTHLDLSH
151  SSFTGLIPSE ICHLSKLHVL RICDQYGLSL VPYNFELLLK NLTQLRELNL
201  ESVNISSTIP SNFSSHLTTL QLSGTELHGI LPERVFHLSN LQSLHLSVNP
251  QLTVRFPTTK WNSSASLMTL YVDSVNIADR IPKSFSHLTS LHELYMGRCN
301  LSGPIPKPLW NLTNIVFLHL GDNHLEGPIS HFTIFEKLKR LSLVNNNFDG
351  GLEFLSFNTQ LERLDLSSNS LTGPIPSNIS GLQNLECLYL SSNHLNGSIP
401  SWIFSLPSLV ELDLSNNTFS GKIQEFKSKT LSAVTLKQNK LKGRIPNSLL
451  NQKNLQLLLL SHNNISGHIS SAICNLKTLI LLDLGSNNLE GTIPQCVVER
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FIGURE 3 CONTINUED

501 NEYLSHLDLS KNRLSGTINT TFSVGNILRV ISLHGNKLTG KVPRSMINCK
551 YLTLLDLGNN MLNDTFPNWL GYLFQLKILS LRSNKLHGPI KSSGNTNLFM
601 GLQILDLSSN GFSGNLPERI LGNLQTMKEI DESTGFPEYI SDPYDIYYNY
651 LTTISTKGQD YDSVRILDSN MIINLSKNRF EGHIPSIIGD LVGLRTLNLS
701 HNVLEGHIPA SFQNLSVLES LDLSSNKISG EIPQQLASLT FLEVLNLSHN
751 HLVGCIPKGK QFDSFGNTSY QGNDGLRGFP LSKLCGGEDQ VTTPAELDQE
801 EEEEDSPMIS WQGVLVGYGC GLVIGLSVIY IMWSTQYPAW FSRMDLKLEH
851 IITTKMKKHK KRY

FIGURE 4

1 CATTTCTTGA TTTCTTCTCT ATCAACATAA CAAGTTTTGA TCATTTTTAG
51 TGCAGAAATG GATTGTGTAA AACTTGTATT CCTTATGCTA TATACCTTTC
101 TCTGTCAACT TGCTTTATCC TCATCCTTGC CTCATTTGTG CCCCGAAGAT
151 CAAGCTCTTT CTCTTCTACA ATTCAAGAAC ATGTTTACCA TTAATCCTAA
201 TGCTTCTGAT TATTGTTACG ACATAAGAAC ATACGTAGAC ATTCAGTCAT
251 ATCCAAGAAC TCTTTCTTGG AACAAAAGCA CAAGTTGCTG CTCATGGGAT
301 GGCGTTCATT GTGACGAGAC GACAGGACAA GTGATTGCGC TTGACCTCCG
351 TTGCAGCCAA CTTCAAGGCA AGTTTCATTC CAATAGTAGC CTCTTTCAAC
401 TCTCCAATCT CAAAAGGCTT GATTTGTCTT TTAATAATTT CACTGGATCA
451 CTCATTTCAC CAAAATTTGG TGAGTTTTCA AATTTGACGC ATCTCGATTT
501 GTCGCATTCT AGTTTTACAG GTCTAATTCC TTCTGAAATC TGTCACCTTT
551 CTAAACTACA CGTTCTTCGT ATATGTGATC AATATGGGCT TAGTCTTGTA
601 CCTTACAATT TTGAACTGCT CCTTAAGAAC TTGACCCAAT TAAGAGAGCT
651 CAACCTTGAA TCTGTAAACA TCTCTTCCAC TATTCCTTCA AATTTCTCTT
701 CTCATTTAAC AACTCTACAA CTTTCAGGCA CAGAGTTACA TGGGATATTG
751 CCCGAAAGAG TTTTTCACCT TTCCAACTTA CAATCCCTTC ATTTATCAGT
801 CAATCCCCAG CTCACGGTTA GGTTTCCCAC AACCAAATGG AATAGCAGTG
851 CATCACTCAT GACGTTATAC GTCGATAGTG TGAATATTGC TGATAGGATA
901 CCTAAATCAT TTAGCCATCT AACTTCACTT CATGAGTTGT ACATGGGTCG
951 TTGTAATCTG TCAGGGCCTA TTCCTAAACC TCTATGGAAT CTCACCAACA

FIGURE 4 CONTINUED

1001 TAGTGTTTTT GCACCTTGGT GATAACCATC TTGAAGGACC AATTTCCCAT

1051 TTCACGATAT TTGAAAAGCT CAAGAGGTTA TCACTTGTAA ATAACAACTT

1101 TGATGGCGGA CTTGAGTTCT TATCCTTTAA CACCCAACTT GAACGGCTAG

1151 ATTTATCATC CAATTCCCTA ACTGGTCCAA TTCCATCCAA CATAAGCGGA

1201 CTTCAAAACC TAGAATGTCT CTACTTGTCA TCAAACCACT TGAATGGGAG

1251 TATACCTTCC TGGATATTCT CCCTTCCTTC ACTGGTTGAG TTAGACTTGA

1301 GCAATAACAC TTTCAGTGGA AAAATTCAAG AGTTCAAGTC CAAAACATTA

1351 AGTGCCGTTA CTCTAAAACA AAATAAGCTG AAAGGTCGTA TTCCGAATTC

1401 ACTCCTAAAC CAGAAGAACC TACAATTACT TCTCCTTTCA CACAATAATA

1451 TCAGTGGACA TATTTCTTCA GCTATCTGCA ATCTGAAAAC ATTGATATTG

1501 TTAGACTTGG GAAGTAATAA TTTGGAGGGA ACAATCCCAC AATGCGTGGT

1551 TGAGAGGAAC GAATACCTTT CGCATTTGGA TTTGAGCAAA AACAGACTTA

1601 GTGGGACAAT CAATACAACT TTTAGTGTTG GAAACATTTT AAGGGTCATT

1651 AGCTTGCACG GGAATAAGCT AACGGGGAAA GTCCCACGAT CTATGATCAA

1701 TTGCAAGTAT TTGACACTAC TTGATCTAGG TAACAATATG TTGAATGACA

1751 CATTTCCAAA CTGGTTGGGA TACCTATTTC AATTGAAGAT TTTAAGCTTG

1801 AGATCAAATA AGTTGCATGG TCCCATCAAA TCTTCAGGGA ATACAAACTT

1851 GTTTATGGGT CTTCAAATTC TTGATCTATC ATCTAATGGA TTTAGTGGGA

1901 ATTTACCCGA AAGAATTTTG GGGAATTTGC AAACCATGAA GGAAATTGAT

1951 GAGAGTACAG GATTCCCAGA GTATATTTCT GATCCATATG ATATTTATTA

2001 CAATTATTTG ACGACAATTT CTACAAAGGG ACAAGATTAT GATTCTGTTC

2051 GAATTTTGGA TTCTAACATG ATTATCAATC TCTCAAAGAA CAGATTTGAA

2101 GGTCATATTC CAAGCATTAT TGGAGATCTT GTTGGACTTC GTACGTTGAA

2151 CTTGTCTCAC AATGTCTTGG AAGGTCATAT ACCGGCATCA TTTCAAAATT

2201 TATCAGTACT CGAATCTTTG GATCTCTCAT CTAATAAAAT CAGCGGAGAA

2251 ATTCCGCAGC AGCTTGCATC CCTCACATTC CTTGAAGTCT TAAATCTCTC

2301 TCACAATCAT CTTGTTGGAT GCATCCCCAA AGGAAAACAA TTTGATTCGT

2351 TCGGGAACAC TTCGTACCAA GGGAATGATG GGTTACGCGG ATTTCCACTC

2401 TCAAAACTTT GTGGTGGTGA AGATCAAGTG ACAACTCCAG CTGAGCTAGA

FIGURE 4 CONTINUED

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2451  TCAAGAAGAG GAGGAAGAAG ATTCACCAAT GATCAGTTGG CAGGGGGTTC
2501  TCGTGGGTTA CGGTTGTGGA CTTGTTATTG GACTGTCCGT AATATACATA
2551  ATGTGGTCAA CTCAATATCC AGCATGGTTT TCGAGGATGG ATTTAAAGTT
2601  GGAACACATA ATTACTACGA AAATGAAAAA GCACAAGAAA AGATATTAGT
2651  GAGTAGCTAT ACCTCCAGGA TTCAAAGATT TCCGAGTTCC CAGTTGCTTG
2701  GGATGCAGAT AAAAGCCTTT TTATCTTTCA TAGTTTCTTA TCCTATGAAT
2751  AAAGATTTTA TTTTCATTTG TCTATGGCAC GTAGATATGT TCCGTCACTA
2801  AAAACATTGT ATTTCTCTCA ACTCTTTCGT CACATGATAT CAAAGAACAC
2851  TTGACTTCAA TTAAGTTAAA AAAAAAAAAA
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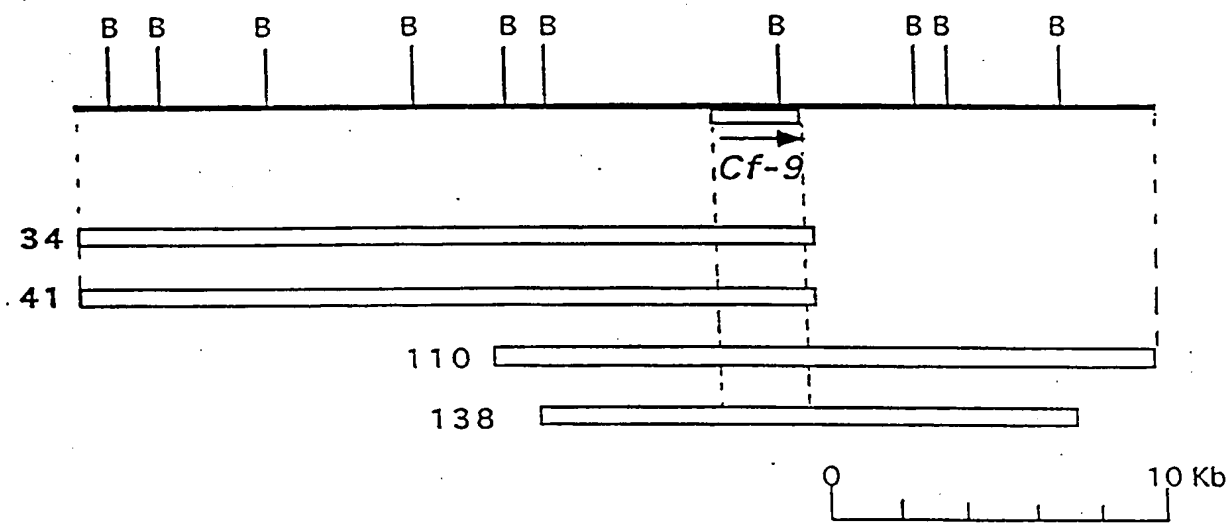
FIGURE 5



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/02812

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/29 C12N15/82 C12N1/21 C12N5/10 A01H5/00
A01H5/10 C12Q1/68 A01N65/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H A01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE PLANT JOURNAL, vol.2, no.3, 1992 pages 367 - 373 TOUBART, P., ET AL. 'Cloning and characterization of the gene encoding the endopolygalacturonase-inhibiting protein (PGIP) of Phaseolus vulgaris L.' see the whole document ---	1
X	PLANT PHYSIOLOGY, vol.102, May 1993 pages 133 - 138 STOTZ, H.U., ET AL. 'Molecular characterization of a polygalacturonase inhibitor from Pyrus communis L. cv Bartlett' see the whole document --- -/--	1

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 May 1995	30.05.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Maddox, A

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 11241 (CONSIGLIO NAZIONALE DELLE RICERCHE) 10 June 1993 see the whole document	1,4-8
P,X	SCIENCE, vol.266, 4 November 1994, LANCASTER, PA US pages 789 - 793 JONES, D.A., ET AL. 'Isolation of the tomato Cf-9 gene for resistance to Cladosporium fulvum by transposon tagging' see the whole document	1-3,6,7
A	MOL. PLANT-MICROBE INTERACT., vol.6, 1993 pages 348 - 357 JONES, D.A., ET AL. 'Two complex resistance loci revealed in tomato by classical and RFLP mapping of the Cf-2,Cf-4,Cf-5, and Cf-9 genes for resistance to Cladosporium-fulvum' see the whole document	1-15
A	CURRENT PLANT SCIENCE AND BIOTECHNOLOGY IN AGRICULTURE, VOL. 10. ADVANCES IN MOLECULAR GENETICS OF PLANT-MICROBE INTERACTIONS, VOL. 1; 5TH INTERNATIONAL SYMPOSIUM ON THE MOLECULAR GENETICS OF PLANT-MICROBE INTERACTIONS, INTERLAKEN, SWITZERLAND., 1991, SEPT. 9-14, 1990. pages 276 - 279 DICKINSON, M., ET AL. 'Strategies for the cloning of genes in tomato for resistance to Fulvia-fulvia' see the whole document	1-15
A	GENET. ENG. (N.Y.), vol.14, 1992 pages 99 - 124 BENNETZEN, J.L., ET AL. 'Approaches and progress in the molecular cloning of plant disease resistance genes' see the whole document	1-15
A	WO,A,91 15585 (RIJKSLANDBOUWUNIVERSITEIT WAGENINGEN) 17 October 1991 see the whole document	1-15
A	SCIENCE, vol.262, 26 November 1993, LANCASTER, PA US pages 1432 - 1436 MARTIN, G.B., ET AL. 'Map-based cloning of a protein kinase gene conferring disease resistance in tomato' see the whole document	1-15

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCIENCE, vol.258, 5 November 1992 pages 985 - 987 JOHAL, G.S., ET AL. 'Reductase activity encoded by the HM1 disease resistance gene in maize' see the whole document -----	1-15

INTERNATIONAL SEARCH REPORT

Inte[illegible]onal Application No
PCT/GB 94/02812

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